AD_____

Award Number: DAMD17-03-1-0304

TITLE: Identification of breast cancer specific proteolytic activities for targeted prodrug activation

PRINCIPAL INVESTIGATOR: Samuel R. Denmeade, M.D.

CONTRACTING ORGANIZATION: Johns Hopkins University
Baltimore, MD 21205-2196

REPORT DATE: May 2006

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01/05/2006	Final	1 May 2003 – 31 Apr 2006

4. TITLE AND SUBTITLE

Identification of breast cancer specific proteolytic activities for targeted prodrug activation

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0304

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Samuel R. Denmeade, M.D.

E-Mail: denmesa@jhmi.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Johns Hopkins University
Baltimore, MD 21205-2196

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT The underlying hypothesis of this proposal is that a breast tissue/cancer proteolytic activity can be identified by screening the extracellular fluid from human breast cancers with a fluorescence quenched random peptide library. The peptide substrate(s) identified from this screening could be used to produce prodrugs that are targeted for specific activation by proteolytic activity present in extracellular fluid of breast cancers while avoiding systemic toxicity. In the first year we developed methods to synthesize large fluorescently quenched peptide libraries as outlined in task 1 and screened for hydrolysis by human glandular kallikrein 2 shown to be present in ~ 75% of breast cancers. A peptide substrate was identified and couple to the thapsigargin analog, L12ADT to produce a prodrug that was readily hydrolyzed by hK2, stable in human plasma in vitro and mouse plasma both in vitro and in vivo, and was selectively cytotoxic to cancer cells in the presence of enzymatically active hK2. These studies demonstrated the feasibility of the approach to identification of protease substrates outlined in tasks 1-3 of the proposal. However, incubation of breast cancer homogenates or concentrated media from breast cancer cell lines did not yield any hydrolyzed peptides. This lack of hydrolysis is most likely is due to a combination of ng/ml concentrations of protease in the extracellular fluid and the need to dilute samples to cover entire bead library (i.e. 10-40 mls). New approaches are needed to identify breast cancer proteases that can concentrate proteases within breast cancer extracellular fluid or which can screen large libraries in much smaller volume. Two such methods, in vitro compartmentalization and macroglobulin complexation are being evaluated in the laboratory.

15. SUBJECT TERMS
Peptide; Prodrug; Combinatorial Library; Targeted Delivery

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	15	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

Page Number

INTRODUCTION:

From a very early stage in the disease process, breast cancers are composed of a heterogeneous collection of cells. These breast cancer cells within a given patient have varying sensitivities to commonly used antiproliferative agents. Metastatic breast cancers are often initially responsive to the commonly used chemotherapeutic agents such as doxorubicin and the taxanes. Unfortunately, no patient is cured by these therapies and thus, metastatic breast cancer is uniformly fatal. New effective therapies for breast cancer are therefore urgently needed.

Currently used chemotherapies are for the most part antiproliferative agents and general cytotoxins that take advantage of the differential rate of growth of cancerous versus normal tissue. These therapies, therefore, are not breast cancer cell specific in their cytotoxicity and their use is often associated with significant dose limiting toxicities. New strategies are needed to inhibit breast cancer specific targets while at the same time avoiding toxicity to normal host tissues. The approach outlined in this proposal is to inhibit a ubiquitous intracellular protein whose function is mandatory for survival of all cell types. This approach would overcome the problem of heterogeneity and "resistance" as all cancer cells within a tumor could be killed via this approach. The obvious shortcoming of this approach is that the cytotoxicity would not be cell-type specific and administration of such a general toxin would be associated with significant systemic toxicity.

Previously we and others have identified such a ubiquitous intracellular protein, the Sarcoplasmic/ Endoplasmic Reticulum Ca^{2+} ATPase (SERCA) pump (1-4). We documented that inhibition of this SERCA pump by the natural plant product thapsigargin (TG) could induce apoptosis of all cell types including breast and prostate cancer cell lines (3-5). Because TG does not possess the primary amine needed for coupling to the C-terminal carboxyl of a peptide carrier, primary amine analogs of TG were made (6,7). Based on a model of the TG binding site within the SERCA pump it was determined that modifications of the TG molecule could possibly be made in the side chain in the 8-position without adversely effecting SERCA pump inhibitory activity (6,7). Using this rationale a series of TG analogs (i.e. ~50) modified in the 8-position with primary amine containing side chains were synthesized by my long time collaborator and discoverer of TG, Dr. Soeren Christensen from the Royal Danish School of Pharmacy in Copenhagen (6,7). These analogs were characterized for their ability to inhibit the SERCA pump and elevate intracellular calcium (6,7). In addition, these analogs were assayed for cytotoxic activity against androgen independent human prostate cancer cells in vitro (6,7). The best of these analogs contained a 12-amino dodecanoate side chain (12ADT) coupled to the amino acid leucine (L12ADT) and was found to have an LD_{50} value against prostate cancer cells of ~ 30 nM. This LD_{50} is identical to that reported for TG (25). In addition, we developed a strategy to target this potent TG analog specifically to sites of prostate cancer to avoid systemic toxicity (8). This approach targets the proteolytic activity of the serine protease prostate-specific antigen (PSA) (8). We identified a PSA specific peptide substrate (9) that is can be coupled to the L12ADT analog of TG to produce an inactive prodrug that can only be proteolytically activated by enzymatically active PSA present within the extracellular fluid prostate cancer (9-11).

Breast cancer cells, like prostate cancer, secrete a variety of proteases. While a PSA-like protease that is secreted in large amounts in a tissue restricted manner has yet to be identified for breast tissue, there are a number of proteases whose expression has been demonstrated to be relatively increased in breast cancer tissue. Examples of such proteases include cathepsins, kallikreins and members of the matrix metalloproteinase family (12-22). Although newer techniques like differential display and SAGE analysis are powerful tools that are yielding large numbers of putative new therapeutic targets, these techniques do not always provide information about functional activity of identified expression products. This functional information is particularly critical when evaluating protease expression because the activity of these proteins is tightly regulated at a number of levels (e.g. expression levels, processing to active protease, binding to inhibitors, auto-degradation). Therefore, additional methods are needed to that will help define not only the presence but also the functional activity of these proteases. The significance of this proposal is that it proposes a strategy to identify specific peptide substrates for breast cancer/tissue specific proteolytic activities. These studies may help to identify new diagnostic and/or therapeutic targets in breast cancer. In addition, the specific peptide substrates will be

incorporated into prodrugs to yield novel targeted therapies for breast cancer while avoiding toxicity to normal tissue.

BODY:

Hypothesis: The underlying hypothesis of this proposal is that a breast tissue/cancer proteolytic activity can be identified by screening the extracellular fluid from human breast cancers with a fluorescence quenched random peptide library. The peptide substrate(s) identified from this screening could be used to produce prodrugs that are targeted for specific activation by proteolytic activity present in extracellular fluid of breast cancers while avoiding systemic toxicity. The peptide substrate(s) will also be used to isolate and characterize specific protease responsible for specific substrate hydrolysis.

To accomplish the goal of the proposal, we outlined 4 tasks that would be completed over the three year funding period. The objective of **Task 1** (months 0-12) was to synthesize fluorescence quenched random peptide libraries to identify proteolytic activities present in the extracellular fluid of human breast cancer cell suspensions. The objective of **Task 2** (months 3-12) is to synthesize soluble fluorescent peptide substrates to characterize specificity and efficiency of hydrolysis. The objective of **Task 3** (months 9-24) is to synthesize thapsigargin prodrugs by coupling thapsigargin analog to lead peptide substrates identified in tasks 1-3. The objective of Task 4 (months 24-36) is to identify specific protease(s) responsible for proteolytic activity using peptide substrates identified in tasks 1-2.

As an initial step toward accomplishing we needed to develop expertise in the synthesis and characterization of large fluorescence quenched random peptide libraries of ~ 1.5- 2 million peptide sequences containing 6 random amino acids. To produce these libraries we used the “one bead-one peptide” splitting and mixing technique (23-24). This approach produces peptides bound to PEGA grafted “beads” in such a way that each individual bead contains many copies of one unique peptide sequence (25). The peptides are synthesized using a previously described approach with a fluorescent molecule [aminobenzoic acid (ABZ)] at the carboxy terminus and a quencher molecule (nitrotyrosine) at the amino terminus (26-29). This design produces a fluorescence quenched library because the emission spectrum of ABZ overlaps exactly with the absorbance spectrum of nitrotyrosine. Cleavage of the peptide sequence by a protease liberates the quencher moiety resulting in a fluorescent bead that is easily visible and easily removed for peptide sequencing, figure 1.

To accomplish task 1 we proposed to incubate these libraries with extracellular fluid from breast cancers. However, prior to exposing fluid from breast cancers that most likely would contain a mixture of proteases, we decided to work out the methodologies for synthesizing and screening a large random library using a more simplified system. Therefore, prior to screening the breast cancer fluid we chose to use the method to identify a substrate for a single purified protease. For these preliminary studies we selected the serine protease human glandular kallikrein 2 (hK2). HK2 has been well-characterized in prostate tissue but is also known to be selectively produced by ~ 75% of breast cancers (Black MH, et al. Expression of a prostate-associated protein, human glandular kallikrein (hK2), in breast tumours and in normal breast secretions. Br J Cancer. 2000;82:361-7).

Through a collaboration with Dr. Hans Lilja at Memorial Sloan Kettering, we have access to large amounts of purified enzymatically active hK2. Therefore, in preliminary studies we generated a fluorescence quenched, combinatorial peptide library and incubated it with hK2. Following incubation, a series of putative peptide substrates were identified and resynthesized as soluble peptide substrates. These soluble substrates were screened for hK2 activity and plasma stability. Finally, the best of these substrates with the sequence Gly-Lys-Ala-Phe-Arg-Arg (GKAFRR) was coupled to a potent analog of thapsigargin to generate the prodrug GKAFRR-L12ADT. This prodrug was then characterized for hydrolysis by hK2, stability in human plasma and selective cytotoxicity against hK2 producing and non-producing cancer cell lines. The rationale, methods and results of these studies using hK2 are described in detail in the attached appendix (appendix 1) which is a manuscript that has been recently submitted for publication.

These studies using purified hK2 allowed our laboratory to develop expertise and techniques required to accomplish goals outlined in tasks 1-3 of the proposal. The results generated using the combinatorial library approach to identify hK2 peptide substrates suggested that a similar approach, as outlined in the proposal, could

successfully yield breast cancer specific substrates. Therefore, a large combinatorial library was synthesized containing ~ 1.5 million random sequences of 6 amino acids in length. The library was screened and a peptide with the sequence Gly-Lys-Ala-Phe-Arg-Arg (GKAFRR) was idenitified that was very efficiently hydrolyzed by hK2 with a Km of 26.5 μM, the k_{cat} at 1.09 sec^{-1} and the k_{cat}/K_m ratio was 41,132 sec^{-1} M^{-1}. This substrate was subsequently attached to a previously identified analog of the potent cytotoxin thapsigargin. The analog, termed L12ADT, is as potent a cytotoxin as thapsigargin but contains an amino acid linker, leucine, which allows for attachment to peptides. On this basis, the prodrug GKAFRR-L12ADT was synthesized. This prodrug is stable to hydrolysis in human plasma. It is more toxic to hK2 producing cell lines than non-hK2 producing cell lines in vitro. Subsequently, the maximally tolerated dose was determined in mice and found to be 6 mg/kg. At this dose, the half life of the drug in mouse plasma is 40 minutes. The prodrug demonstrated significant antitumor effect in vivo while it was being administered, but prolonged intravenous administration was not possible due to local toxicity to tail veins.

On the basis of these results we will screen the prodrug against wild type breast cancer cell lines that produce varying levels of hK2. We will also construct breast cancer cell lines that overexpress hK2 to generate models that can be used for in vivo testing.

Studies with Human Breast Cancer extracts

Our original intention was to incubate this library with extracellular fluid obtained from fresh breast cancer specimens obtained directly from the operating room. Unfortunately, over the course of this first funding year, we have been unable to obtain such fresh samples from the Johns Hopkins Department of Pathology as anticipated. Instead, we have had to rely on frozen specimens to complete these screening assays. On this basis, the newly synthesized combinatorial library was initially incubated with homogenate from 2 breast cancer specimens (total of ~ 200 mg of cancer tissue). This homogenate was incubated with 25% of the peptide library, initially for a period of 48 hrs. At this point in time, no beads had become fluorescent, indicating that no hydrolysis had occurred. The library was incubated for a total of 5 days, again with no positive fluorescent beads.

One possible reason for lack of hydrolysis of peptide containing beads in this assay may be due to small amount of frozen cancer tissue that was available for the assay which, concomitantly, would contain low amounts of proteases. For this assay we obtained homogenized tissue in 1 ml of buffer. The peptide library of ~1 million beads requires ~ 40 ml of solution to wet all of the beads. Thus, the breast cancer homogenate had to be diluted ~ 10-fold more just to cover the beads in 25% of the library. Thus, low levels of protease present in the extracellular fluid would be diluted to levels that may lie below sensitivity of assay, even after 5 days incubation. For example, hK2 is present in media of prostate cancer cells at levels of ~ 10-50 ng/ml. Dilution of this media ~ 10-fold would yield a concentration of hK2 of ~ 1- 5 ng/ml. In the combinatorial library screen a level of 4 μg/ml hK2 was required to identify hydrolyzed peptides over 48 hr incubation period. In the original proposal our plan was to use media from single cell suspension of human breast cancers that had been conditioned for multiple days as the source of extracellular protease. Due to the difficulty in obtaining such fresh specimens we opted to use a human breast cancer cell line, MCF-7, to determine if this combinatorial peptide methodology could be used to identify proteolytic activity. For these assays, MCF-7 cells were grown in standard serum containing media until cells were ~60% confluent. Cells were then transferred into DMEM media without any additional serum to obtain conditioned media that did not have any serum protease inhibitors present. Media was conditioned for 4 days and then media from 5 T-75 flasks (i.e. ~ 5 x 10^7 cells) was collected and concentrated ~8-fold to a volume of 10 ml. Again, this fluid was incubated with beads representing ~ 25% of the library (i.e. ~ 250,000 peptide sequences). After 5 days incubation, 3 fluorescent beads were observed, but Edman sequencing revealed that these beads represented false positives that had not been correctly synthesized.

On the basis of the preliminary studies with hK2 and these two experiments with frozen breast cancer tissue and concentrated conditioned media from a human breast cancer cell line, we have concluded that the combinatorial bead strategy for identifying breast cancer proteases has a number of limitations and will need to be modified in future studies. The major limitations of this approach are:

1. The beads used for solid phase synthesis are ~ 300 micrometers in diameter and therefore, one million beads takes up ~ 40 ml volume. This requires either a large amount of extracellular fluid (ECF) or dilution of ECF multiple-fold to cover all of the beads.

2. The proteases in the ECF of breast cancer cells most likely are in concentrations in the ng/ml range and this bead methodology appears to work best with proteases in the μg/ml range.

3. Proteases greater than ~ 35,000 MW do not easily access the central portions of the beads, resulting in limited hydrolysis and poor sensitivity.

4. Steric constraints imposed by the solid phase surface limit peptide hydrolysis compared to soluble peptide substrates.

Studies for 2005-2006

On the basis of our prior studies it is clear that future success in the project requires development of methods to (1) decrease the physical size of the library; (2) to screen soluble peptides in smaller volume of fluid that can be obtained from ECF of human breast cancers; (3) or to develop alternative method to enrich for breast cancer specific proteases. To accomplish the first two tasks we have had to modify the combinatorial peptide strategy originally outlined in task 1 because this approach requires large volume of fluid due to physical size of the bead library. We are exploring two different approaches to accomplish this taks. The first uses the combinatorial screening method described by Sepp, et al. depicted in Figure 1 (30). In this approach, an oligonucleotide is generated that encodes a random peptide flanked by peptide tags recognized by commercially available antibodies. This oligonucleotide is coupled to biotin and then attached to a streptavidin coated microbead (i.e. one micron diameter). Beads are then compartmentalized in a water-oil emulsion to give on average ~ 1 bead per compartment and are transcribed and translated in the compartment. For our application the gene will encode for a peptide with the general sequence FLAG Tag- Random 8 amino acid Peptide- HA Tag. Peptides will be bound to bead by binding to commercially available biotinylated antibody to FLAG attached to streptavidin surface. Beads are then incubated with protease containing ECF from breast cancers. Non-hydrolyzed beads will bind to commercially available HRP-linked HA antibody and will become fluorescent. Beads containing peptides that have been hydrolyzed by proteases will not bind to HRP-linked HA antibody and will not be fluorescent. These beads can then be rapidly sorted on flow cytometer. Non-fluorescent beads can be captured and nucleotide sequence determined to then determine random peptide sequence. These peptides can then be resynthesized as soluble peptides and rescreened as outlined in task 2. Further modifications to peptide sequence can be made suing this approach to identify an optimal peptide for protease activity present in ECF.

There are many advantages to the In Vitro Compartmentalisation approach for this application. First, the physical volume of the library is greatly reduced by decreasing the size of the bead particles from 300 to 1 micron in diameter. A library of ~ one million sequences will only take up ~50-100 μl volume in this approach (31). In addition, the use of an oligonucleotide to generate the peptide will allow for rapid modification and optimization of peptide sequences. The time to determine peptide sequence and costs of sequencing will be greatly reduced by this approach. Finally, bead screening and sorting can be performed using standard flow cytometer available as a core facility within the Oncology Department. This method, therefore, allows for rapid screening of large numbers of beads in a short period of time.

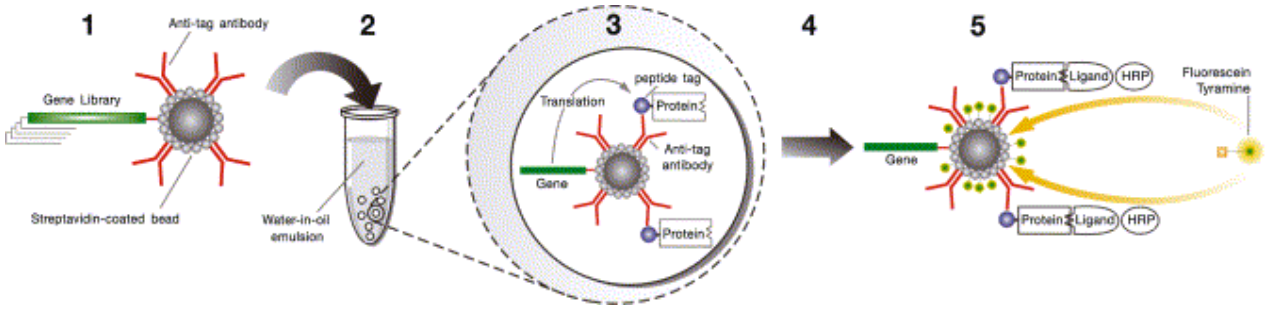


Figure 1. Creation of microbead display libraries by In Vitro Compartmentalisation and selection for binding using flow cytometry. A repertoire of genes encoding protein variants, each with a common N- or C-terminal epitope tag, are linked to streptavidin-coated beads carrying antibodies that bind the epitope tag at, on average, ≤1 gene per bead (1). The beads are compartmentalised in a water-in-oil emulsion to give, on average, <1 bead per compartment (2), and transcribed and translated in vitro in the compartments. Consequently, in each compartment, multiple copies of the translated protein become attached to the gene that encodes it via the bead (3). The emulsion is broken (4), and the microbeads carrying the display library isolated. The beads are incubated with ligand coupled to horseradish peroxidase (HRP), washed to remove unbound ligand and incubated with hydrogen peroxide and fluorescein tyramide (5). Immobilised HRP converts the fluorescein tyramide into a short-lived, free-radical intermediate which reacts with adjacent proteins. Hence, beads displaying proteins that bind ligand become labeled with multiple fluorescein molecules. These beads can then be enriched (together with the genes attached to them) by flow cytometry. [Figure and legend from Sepp et al. FEBS Letters 532:455-458, 2002 (ref 30].

Over the past year we created components of the In Vitro Compartmentalisation system. These include the construction of the gene library encoding for peptide that would include the appropriate binding tags (i.e. FLAG, etc). We are currently trying to develop the methods to generate the water-in-oil emulsion system to capture individual gene sequences. We will begin testing to determine if we can achieve adequate translation to produce enough peptide in the individual microsomes for detection by fluorescence based cell sorting. Once we have optimized the system we will begin to analyze extracts from breast tissues and conditioned media from breast cancer cell lines to determine if this In Vitro Compartmentalisation system is applicable to the goal of identifying breast cancer specific peptide substrates.

A second approach to decreasing the size of the library and to screen soluble peptides in smaller volume of fluid that can be obtained from ECF of human breast cancers is to use a phage display approach. To accomplish this, we have designed a “Substrate Phage” approach which was originally described by Matthews and Wells in 1993 (32). This group had constructed a fusion protein of a variant of human growth hormone as the amino-

terminal domain of the phage coat protein used to bind to an affinity support, followed by a randomized protease substrate sequence and the carboxyl-terminal domain of M13 gene III. Each fusion protein was displayed as a single copy on filamentous phagemid particles (substrate phage) (32). The random piece was flanked on either side by a linker sequence used to break secondary structure imposed by protein and to provides flexibility for the protease to bind and cleave the substrate sequence. This group demonstrated that three rounds of panning were sufficient to obtain a consensus sequence among the preferred cleavage sites. Since then a large number of groups have used this or a variant of this approach for identification of protease peptide substrates (33,34). In our own laboratory we have used phage based approach to identify peptides that bind selectively to the prostate tissue specific carboxypeptidase Prostate Specific Membrane Antigen (PSMA) (35).

Substrate phage can be created either as a monovalent or as pentavalent display (34). Both approaches have their own advantages and disadvantages (reviewed in 34). For our design we chose to make a pentavalent display of the random peptide substrate library. Our design of the substrate phage incorporates the following elements.

1. Flag peptide
2. Linker 1
3. Random 6-mer substrates
4. Linker 2

DYKDDDDKSSGGSG**XXXXXX**GGGGGS

Flag tag Linker 1 Random Insert Linker 2

Schematic of phage substrate design

We selected the FLAG tag because of its small size producing less steric hindrance for the protease and the availability of very high affinity anti-Flag monoclonal antibodies. Linker 1 and 2 were chosen based on the previously used sequence by Smith et al (36).

Using the method described below we generated a small scale library of 10^5 phage and selected 10 random plaques for sequencing to confirm the presence of tag, linkers and random insert. The following 10 sequences were obtained from this selection:

1. VVPFYSHSDDYKDDDDKSSGGSGYSLKVTGGGGGS…
2. VVPFYSHSDDYKDDDDKSSGGSGRKCHLVGGGGGS…
3. VVPFYSHSDDYKDDDDKSSGGSGVMAKLTGGGGGS…
4. VVPFYSHSDDYKDDDDKSSGGSGDRVNVMGGGGGS…
5. VVPFYSHSDDYKDDDDKSSGGSGYSAVMPGGGGGS…
6. VVPFYSHSDDYKDDDDKSSGGSGYVYLFFGGGGGS…
7. VVPFYSHSDDYKDDDDKSSGGSGVTISSMGGGGGS…
8. VVPFYSHSDDYKDDDDKSSGGSGAVSQTLGGGGGS…
9. VVPFYSHSDDYKDDDDKSSGGSGFNFRYMGGGGGS…
10. VVPFYSHSDDYKDDDDKSSGGSGFYRSRWGGGGGS…

The sequence VVPFYSHSD is also shown here to confirm the presence of the signal sequence which is needed to transport the fusion protein to the periplasm of E.coli. This signal peptide is cleaved after transport. The signal sequence also confirms that the sequences were cloned in frame. DYKDDDDK is sequence of the FLAG peptide. The random inserts are underlined

As a positive control we have used the same tag and linkers to clone Factor Xa protease cleavage sequences into the phage in order to optimize the FAP cleavage and selection protocols. To generate this control the sense oligo used was 5'/Phos GTACCTTTCTATTCTCACTCTGACTACAAAGACGATGACGACAAGTCGAGCGGTGGTAGTGGTTCTGGTATC GAGGGAAGGGGTGGTGGTGGTGGTTC - 3'. Plaques were picked and sequenced to determine the presence of Factor Xa cleavage site SGIEGR. Sequencing of two plaques yielded:

VVPFYSHSDDYKDDDDKSSGGSGSGIEGRGGGGGS…

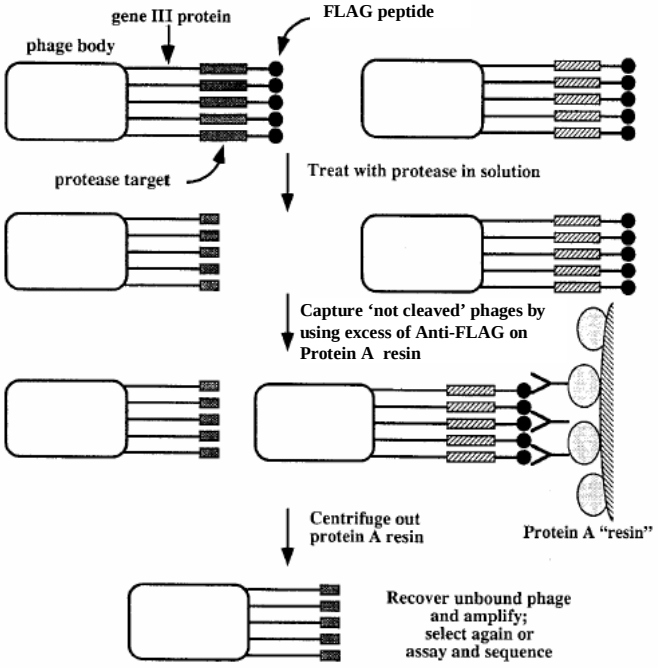
Currently we are using this clone to develop optimal assay using methodology outlined below prior to large scale screening of phage library with FAP.

The protocol and methods described below will be scaled up to generate a library of 10^8 phage. Two approaches will then used for screening the library.

1. "Solution-Phase": Pre-incubation of phage library with ECF from human breast cancer followed by capture of 'non cleaved' phage using excess of Anti-FLAG antibody on protein A resin. Phages left in solution (i.e. Protease cleaved Flag tag) will be amplified and used for subsequent rounds of selection. Typically three rounds of selection are used to select sequences with adequate diversity.
2. "Solid-Phase": Pre-incubation of library with Anti-FLAG antibody on protein A resin, followed by incubation with Breast Cancer ECF. Protease-released phages can be easily separated and used for further selection rounds by centrifugation and capture of non-cleaved phage remaining on Protein A resin.

The Solution-Phase approach has the advantage of presenting the substrate sequence to the protease in a more accessible manner (because it has more degrees of freedom when it is free in solution compared to being immobilized on a solid surface). The only disadvantage of this approach is that it sometimes requires more rounds of selection as the depletion of 'non cleaved' phage in each round is not 100% efficient. The Solid-Phase approach has the advantage that it gives fewer "hits" but these hits are more likely to be the most favorable ones. Both approaches have been used in literature. Since panning of the library takes less than a week we will perform screening in parallel using both approaches.

A schematic version of the Solution-Phase approach [adapted from Smith et al (36)] is shown below:



After three rounds of selection, phage will be analyzed to determine the amino acid sequence of the random inserts. Analyses will be performed to evaluate whether consensus sequences are present. Peptides from this phage analysis will be resynthesized and tested for hydrolysis in breast cancer ECF extracts to determine best peptide sequence for further development

KEY RESEARCH ACCOMPLISHMENTS:

1. Synthesized and characterized a large fluorescently quenched combinatorial peptide library and successfully identified a substrate for the serine protease human glandular kallikrein 2 (hK2), which is expressed by human prostate cancers and by ~ 50% of human breast cancers.
2. Characterized hK2 hydrolysis of soluble hK2 substrates and determined plasma stability
3. Synthesized a hK2-activated thapsigargin prodrug that is efficiently hydrolyzed by hK2 and stable in human plasma
4. Demonstrated that this hK2 prodrug is selectively toxic in the presence of enzymatically active hK2 in vitro.
5. Completed prodrug distribution studies in vivo using radiolabeled prodrug.
6. Completed pharmacokinetic studies in vivo using hK2 activated prodrug
7. Screened homogenates of frozen human breast tumors and conditioned media from the human breast cancer cell line MCF-7 but did not identify a putative peptide substrate for a breast cancer protease.
8. Designed random gene library to be used in In Vitro Compartmentalization studies
9. Generate FLAG-tagged phage display library for screening

REPORTABLE OUTCOMES:

Presentations:

Janssen S, **Denmeade SR.** Identification of Tumor Associated Protease Substrates Using Combinatorial Chemistry. AACR/EORTC Molecular Targets and Cancer Therapeutics Meeting, Frankfurt, Germany 2002.

Manuscripts and Abstracts:

1. Janssen S, **Denmeade SR.** Identification of Tumor Associated Protease Substrates Using Combinatorial Chemistry. Eur J Cancer 38 Suppl 7:S97, 2002.
2. Janssen S, Jakobsen CM, Rosen DM, Reineke U, Christensen SB, Lilja H, **Denmeade SR**. Screening a combinatorial peptide library to develop a human glandular kallikrein-2 activated prodrug as targeted therapy for prostate cancer. Mol Cancer Ther. 2004;3:1439-50**.**
3. Janssen S, Rosen DM, Ricklis RM, Dionne CA, Lilja H, Christensen SB, Isaacs JT, **Denmeade SR**. Pharmacokinetics, Biodistribution and Antitumor Efficacy of a Human Glandular Kallikrein 2 (hK2) - Activated Thapsigargin Prodrug. Prostate, In press, 2005
4. Lebeau A, Janssen S, **Denmeade SR.** Identification of breast cancer specific proteolytic activities for targeted prodrug activation. Proceeding, Era of Hope Meeting, 2005;P 67-4.
5. Aggarwal S, Singh P, Topaloglu O, Isaacs JT, **Denmeade SR**. A dimeric peptide that binds selectively to prostate-specific membrane antigen and inhibits its enzymatic activity. Cancer Res. 2006;66:9171-7.

Support for Post-Doctoral Fellow, Dr. Samuel Janssen to carry out experiments outlined in this proposal from May 1, 2003 to August 30, 2003. Dr. Janssen subsequently obtained a senior staff scientist position at Amylin, Inc., San Diego, CA.

Salary support for graduate student, Aaron LeBeau, beginning 1/1/04 to complete tasks 1-3 outlined in proposal.

CONCLUSIONS:

At the end of three years of funding we have developed methods to synthesize large fluorescently quenched peptide libraries as outlined in task 1. In preliminary studies, we incubated these libraries with a purified serine protease, human glandular kallikrein 2, and identified a series of putative substrates. These substrates were resynthesized as soluble peptides and characterized for hK2 hydrolysis and plasma stability. Peptides were then identified that were excellent hK2 substrates but unstable to non-specific hydrolysis in human plasma. Coupling these peptides to the thapsigargin analog, L12ADT, however, produced a prodrug that was readily hydrolyzed by hK2, stable in human plasma in vitro and mouse plasma both in vitro and in vivo, and was selectively cytotoxic to cancer cells in the presence of enzymatically active hK2. The hK2 substrate was tested in vivo and showed some antitumor efficacy, however, improved formulations are required to allow for sustained delivery of the compound. These studies demonstrated the feasibility of the approach to identification of protease substrates outlined in tasks 1-3 of the proposal. However, incubation of breast cancer homogenates or concentrated media from breast cancer cell lines did not yield any hydrolyzed peptides. This lack of hydrolysis most likely is due to combination of ng/ml concentrations of protease in extracellular fluid and need to dilute samples to cover entire bead library (i.e. 10-40 mls). A new approach for combinatorial screening is required that would yield libraries of small physical volume to screen small volume of extracellular fluid from breast cancers without requiring substantial dilution. To accomplish this, we are developing methods to adapt and apply the recently described combinatorial technique of in vitro compartmentalization that allows for rapid screening of large libraries in volumes of < 100 μl for this specific application. This method has been used to screen for binding moieties, but has never been used to screen for proteolytic substrates. In addition, as an alternative approach to screening in small volumes we have recently generated a random phage library for use in screening proteolytic activity in human breast cancer extracellular fluid.

REFERENCES

1. Rasmussen, U., Christensen, S. B., and Sandberg, F. Thapsigargine and thapsigargicine, two new liberators from Thapsia garganica. L. Acta Pharm. Suec., 15: 133-140, 1978.
2. Thastrup, O., Cullen, P. J., Drøbak, B. K., Hanley, M. R., and Dawson, A. P. Thapsigargin, a tumor promoter, discharges intracellular Ca2+ stores by specific inhibition of the endoplasmic reticulum Ca2+-ATPase. Proc. Natl. Acad. Sci. USA, 87: 2466-2470, 1990.
3. Furuya, Y., Lundmo, P., Short, A. D., Gill, D. L., and Isaacs, J. T. The role of calcium, pH, and cell proliferation in the programmed (apoptotic) death of androgen-independent prostatic cancer cells induced by thapsigargin. Cancer Res., 54: 6167-6175, 1994.
4. Lin XS, Denmeade SR, Cisek L, Isaacs JT: The role of growth arrest in programmed (apoptotic) death of prostate cancer cells by thapsigargin. Prostate 33:201-207, 1997.
5. Jackisch, C., Hahm, H., Tombal, B., McCloskey, D., Butash K., Davidson, N., and Denmeade, S.R. Delayed micromolar elevation in intracellular calcium precedes induction of apoptosis in thapsigargin-treated breast cancer cells. Clin Cancer Res 6:2844-2850, 2000.
6. Christensen, S.B., Andersen, A., Kromann, H., Treiman, M., Tombal, B., Denmeade, S.R, and Isaacs, J.T. Thapsigargin Analogues for Targeting Programmed Death of Androgen-Independent Prostate Cancer Cells. Bioorg. Medicinal Chemistry, 7:1273-80, 1999.
7. Jakobsen CM, Denmeade SR, Isaacs JT, Olsen CE, Christensen SB. Design, synthesis and pharmacological evaluation of thapsigargin analogs for targeting apoptosis to prostatic cancer cells. J Med Chem 44: 4696-4703, 2001.
8. Denmeade SR, Isaacs JT. Enzymatic activation of prodrugs by prostate-specific antigen: targeted therapy for metastatic prostate cancer. Cancer J Sci Am 4 Suppl 1:S15-21, 1998.
9. Denmeade, S.R., Lou, W., Malm, J., Lovgren, J., Lilja, H., and Isaacs, J.T. Specific and Efficient Peptide Substrates for Assaying the Proteolytic Activity of Prostate Specific Antigen. Cancer Res., 57:4924-4930, 1997.
10. Denmeade SR, Nagy A, Gao J, Lilja H, Schally A, Isaacs JT. Enzymatic activation of a doxorubicin-peptide prodrug by prostate-specific antigen. Cancer Res., 58:2537-2540, 1998.
11. Khan SR, Denmeade SR. In vivo activity of a PSA-activated doxorubicin prodrug against PSA-producing human prostate cancer xenografts. Prostate 45:80-83, 2000.
12. Duffy MJ, Maguire TM, Hill A, McDermott E, O'Higgins N. Metalloproteinases: role in breast carcinogenesis, invasion and metastasis. Breast Cancer Res 2:252-257, 2000.
13. Lebeau A, Nerlich AG, Sauer U, Lichtinghagen R, Lohrs U. Tissue distribution of major matrix metalloproteinases and their transcripts in human breast carcinomas.
14. Rudolph-Owen LA, Matrisian LM. Matrix metalloproteinases in remodeling of the normal and neoplastic mammary gland. J Mammary Gland Biol Neoplasia 3:177-189, 1998.
15. Jones JL, Glynn P, Walker RA. Expression of MMP-2 and MMP-9, their inhibitors and the activator MTI-MMP9 in primary breast carcinomas. J Pathol 189:161-168, 1999.
16. Yousef GM, Obiezu CV, Luo L-Y,, Black MH, Diamandis EP. Prostase/KLK-L1 is a new member of the human kallikrein gene family, is expressed in prostate and breast tissues and is hormonally regulated. Cancer Res 59:4252-4256, 1999.
17. Chang A, Yousef GM, Scorilas A, Grass L, Sismondi P, Ponzone R, Diamandis EP. Human kallikrein 13 (KLK13) expression by quantitative RT-PCR: an independent indicator of favorable prognosis in breast cancer. Br J Cancer 86:1457-1464, 2002.
18. Black MH, Magklara A, Obiezu C, Levesque MA, Sutherland DJ, Tindall D. Expression of a prostate-associated protein, human glandular kallikrein (hK2), in breast tumors and in normal breast secretion. Br J Cancer 82:361-367, 2000.
19. Yousef GM, Magklara A, Diamandis EP. KLK12 is a novel serine protease and a new member of the human kallikrein gene family-differential expression in breast cancer. Genomics 69:331-341, 2000.
20. Ren WP, Sloane BF. Cathepsins D and B in breast cancer. Cancer Treat Res 83:325-352, 1996.

21. Lah TT, Kalman E, Najjar D, Gorodetsky E, Brennan P, Somers R, Daskal I.Cells producing cathepsins D, B, and L in human breast carcinoma and their association with prognosis. Hum Pathol 31:149-160, 2000.
22. Lin, C-Y., Wang, J-K, Torri, J., Dou, L., Sang, Q. A., and Dickson, R.B. Characterization of a novel, membrane-bound, 80-kDa matrix-degrading protease from human breast cancer cells. J Biol Chem 272:9147-9152, 1997.
23. Lam KS, Salmon SE, Hersh EM, Hruby VJ, Kazmierski WM, Knapp RJ. A new type of synthetic peptide library for identifying ligand-binding activity. Nature 354:82-84, 1991.
24. Lam KS, Lebl M. Synthesis of a one-bead one-compound combinatorial peptide library. Methods Mol Biol 87:1-6, 1998
25. Renil M, Ferreras M, Delaisse JM, Foged NT, Meldal M. PEGA supports for combinatorial peptide synthesis and solid-phase enzymatic library assays. J Pept Sci 4:195-210, 1998.
26. Matayoshi ED, Wang GT, Krafft GA, Erickson J.Novel fluorogenic substrates for assaying retroviral proteases by resonance energy transfer. Science 247:954-958, 1990.
27. Chagas JR, Juliano L, Prado ES. Intramolecularly quenched fluorogenic tetrapeptide substrates for tissue and plasma kallikreins. Anal Biochem 192:419-425, 1991.
28. Angliker H, Neumann U, Molloy SS, Thomas G. Internally quenched fluorogenic substrate for furin. Anal Biochem 224:409-412, 1995.
29. St Hilaire PM, Willert M, Juliano MA, Juliano L, Meldal M. Fluorescence-quenched solid phase combinatorial libraries in the characterization of cysteine protease substrate specificity. J Comb Chem 1:509-523, 1999.
30. Sepp A, Tawfik DS, Griffiths AD. Microbead display by in vitro compartmentalisation: selection for binding using flow cytometry. FEBS Lett 532:455-458, 2002.
31. Griffiths AD, Tawfik DS. Directed evolution of an extremely fast phosphotriesterase by in vitro compartmentalization. EMBO J 22:24-35, 2003.
32. Matthews DJ, Wells JA. Substrate phage: selection of protease substrates by monovalent phage display. Science 1993;260:1113-1117.
33. Deng, S.J. et al. Substrate specificity of human collagenase 3 assessed using a phage-displayed peptide library. J Biol Chem 2000;275;31422-31427.
34. Sedlacek R, Chen E. Screening for protease substrate by polyvalent phage display. Comb Chem High Throughput Screen. 2005;8:197-203.
35. Aggarwal S, Singh P, Topaloglu O, Isaacs JT, Denmeade SR. A dimeric peptide that binds selectively to prostate-specific membrane antigen and inhibits its enzymatic activity. Cancer Res. 2006;66:9171-7.
36. Smith MM, Shi L, Navre M.Rapid identification of highly active and selective substrates for stromelysin and matrilysin using bacteriophage peptide display libraries. J Biol Chem. 1995;270:6440-9.